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(54) Title: METHOD OF INDUCING NITROGEN FIXATION IN PLANTS

(57) Abstract

The invention relates to a method of inducing nitrogen fixation in non-leguminous plants by inoculating the plant with a nitrogen-fixing bacterium, wherein the bacterium is exposed to a nod-factor inducing agent. The invention also relates to a composition comprising a non-leguminuous plant seed mixed with a plant growth medium and an inoculum of a nitrogen-fixing bacterium exposed to an agent capable of inducing nod-factor production in the bacterium. In another aspect the invention relates to a plant growth medium for a non-leguminous plant comprising a bacterium capable of fixing nitrogen wherein the bacterium has previously been exposed to a nod-factor inducing agent.

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METHOD OF INDUCING NITROGEN FIXATION IN PLANTS

The invention relates to plant biotechnology and particularly to the stimulation of nitrogen-fixation in non-leguminous plants by interaction with nitrogen-fixing bacteria.

Legumes, such as clover, have nodules containing nitrogen-fixing bacteria on their roots which fix atmospheric nitrogen so that it is available to the plant as a nutrient. Apart from the commercially unimportant *Parasponia* genus of plants (tropical shrubs in the elm family), non-legumes do not naturally form such nodules. It would be desirable to enable commercially important non-leguminous cereal crops such as wheat, rice, maize, barley, millet, sorghum and rice; tomatoes and other horticulturally important crops, and especially oilseed rape (*Brassica napus* and other *Brassica* species) to fix nitrogen as this would offer the possibility of reducing the amount of nitrogenous fertilizers used at present. Indeed Cocking *et al* ((1994) Agro-Food Industry Hi-Tech Jan/Feb 21-24) remarked that "The introduction of symbiotic biological nitrogen fixation into the major non-legume crops of the world would be one of the most significant contributions biotechnology could make to agriculture.".

Nodulation of legumes by the nitrogen-fixing bacteria rhizobia is generally considered to be controlled by rhizobia nodulation (*nod*) genes. These *nod* genes encode enzymes involved in the synthesis of *Nod* factors that induce morphological changes in legume roots. The four *nod* genes A,B,C and D are the minimum number of genes required for the formation of such extracellular metabolites which induce the root hair curling response in legumes. It has been demonstrated that the expression of some of the rhizobia symbiotic genes with the associated production of rhizobia *Nod* factors, can induce changes in the growth of rice root hairs comparable to

the curling and distortion observed in root hair of legumes during interaction with rhizobia.

Some naturally occurring rhizobia induce nitrogen-fixing root nodules on a wide range of legumes. For instance, strain NGR234 nodulates many legumes, including species in the genera *Arachis, Glycine, Lablab* and *Vigna*. Additionally, this wide host range strain NGR234 induces effective root nodules on the non-legume *Parasponia*. This unique association has prompted a genetic analysis of the nitrogen fixing symbiosis between *Parasponia* and rhizobia. The infection of *Parasponia* by rhizobia requires the nod regulatory gene, together with host-specific nodulation (*hsn*) genes. Thus, nodulation of *Parasponia* requires the co-ordinated expression of *nod*A,B and C genes together with rhizobia *hsn* genes under the regulation of the *nod*D product. This knowledge enabled the construction of a new rhizobium strain (ANU536) which possessed multiple copies of the *nod*D gene on a high copy number vector. This strain was able to induce nodule-like structures on the roots of rice seedlings at a low frequency of 0.1-0.25%. These nodule-like structures were small, white and contained membrane encapsulated bacteria; one of these structures was observed to have an internal structure resembling that found within legume root nodules. However, nitrogen fixation activity has not been detected.

A wide range of nod genes have so far been identified in rhizobia (see Werner D, *Symbiosis of Plants and Microbes* (1992), published Chapman & Hall, especially pages 68 and 69 and Table 3.7 below). However, many of the nod factors have not been characterised.

From Werner (1992): **Table 3.7** Nodulation (*nod*) genes in *Rhizobium* (R), *Bradyrhizobium* (B), *Sinorhizobium* (S) and *Azorhizobium* (A).

Gene	*Functions of gene products*
*nod*ABC	Essential for root hair curling and cortical cell division. *Nod*ABC likely synthesize a phytohormone-like substance. *R. meliloti*: Rmnod-1 identified as a *N*acyl-tri-*N*-acetyl-β-1,4-D-glucosamine tetrasaccharide bearing a sulphate group on carbon 6 of the reducing sugar moiety.
*nod*D1,D2,D3	Transcriptional regulator required for the expression of the other nod genes.
*nod*E	Host range, reported sequence similarity to the *fab*B gene of *E. coli*, condensing enzyme of fatty acid synthase. Membrane protein. Mutants lacking NodE are affected in capsular polysaccharide biosynthesis.
*nod*F	Host range, reported sequence similarity to acyl carrier protein. *Nod*F carries 4'-phosphopantetheine as a prosthetic group. At least two different forms of NodF can be isolated from a NodFe-overproducing strain of *R. leguminosarum* bv. *viciae*.
*nod*G	Host range, reported sequence similarity to dehydrogenases.
*nod*H	Host range, many modify factor produced by *Nod*ABC. may function as a sulphate transferase for the synthesis of NodRm-I.
*nod*I	Reported sequence similarity to ATP binding transport proteins. *R. leguminosarum* bv. *trifolii* mutants defective in *Nod*I have a decrease in the 3-hydroxybutyrate substitution of the capsular polysaccharide.
*nod*J	Membrane protein, may act in conjunction with *nod*I.
*nod*K	Reported only in *B.* sp. (*Parasponia*). Function unknown.
*nod*L	Host range, reported sequence similarity to acetyltransferase.
*nod*M	Host range, reported sequence similarity to amidotransferase.
*nod*N	Reported. Involved in biosynthesis of hair deformation factor.
*nod*O	Function unknown, encoded protein is exported. Reported to bind calcium.
*nod*P	Function unknown, gene hybridizes to *E. coli* genomic DNA.
*nod*Q	Host range, reported sequence similarity to GTP binding proteins, EF-tu and EF-1. May modify factor produced by *Nod*ABC.

*nod*R	Reported only in *R. leguminosarum* bv. *trifolii*. Host range.
*nod*S	Function unknown. MW 23 kDa.
*nod*T	Host range. Function unknown.
*nod*U	Function unknown. MW 62 kDa.
*nod*V	Reported only in *B. japonicum*. Important for host range, sequence similarity to membrane sensor family.
*nod*W	Reported only in *B. japonicum*. Important for host range, sequence similarity to family of transcriptional regulatory proteins.
*nod*X	Reported only in *R. leguminosarum* strains capable of nodulating Afghanistan pea.
*nod*Y	Reported only in *B. japonicum*, but found in all rhizobia by hybridization. Function unknown.
*nod*Z	Host range required for growth under microaerobic conditions.
*nod*1A	Host range. Essential for nodulation of selected soybean cultivars. Reported only from *B. japonicum*. Shows sequence similarity to MerR, a transcriptional regulatory protein.
*nol*D, *nol*E, *nol*F, *nol*G	Host range in *R. meliloti*.

Expression of nod genes may be regulated in legumes by inducers such as the flavones or flavonones exuded by legume roots.

It is known that the flavonoid naringenin increases root nodulation and nitrogen fixation when added to the rhizosphere during inoculation of alfalfa cultivar Rijka T9 with *Rhizobium meliloti* (Jain *et al* (1990) World J Microbiol 6:434-436). However, attempts to extend these observations to other alfalfa cultivars did not increase nodulation (Phillips (1992) Phenolic Metabolism in Plants, Chapter 7, page 219, Ed. Stafford and Ibraheim, Plenum Press, New York) which strongly suggests that the observations could not be extended to other legumes.

The present invention aims to provide a method for inducing nitrogen

fixation in non-legume plants.

Various aspects of the present invention are defined in the accompanying claims.

According to the invention there is provided a method of inducing nitrogen fixation in a non-leguminous plant by inoculating the plant with a nitrogen-fixing bacterium; wherein the bacterium is exposed to a nod-factor inducing agent.

The term "inoculating" is intended to embrace any means by which the plant and bacterium are brought into nitrogen-fixing interaction.

Unexpectedly, the inventor has found that exposure of the bacterium to a nod-factor inducing agent in the method of the invention induces the bacterium to produce *Nod* factors which stimulate the entry of the bacterium into the non-leguminous plant root system and nitrogen fixation.

The results presented herein demonstrate for the first time that nod-factor inducing agents such as flavonoids can stimulate the interaction of nitrogen fixing bacteria with a non-legume crop, such as wheat, resulting in nitrogen fixation.

In a further aspect the invention provides a non-leguminous plant in a nitrogen fixing interaction with a bacterium obtainable by the method of the invention.

The phrase "nitrogen-fixing interaction" is intended to include any plant/bacterial interaction in which atmospheric nitrogen is made available to the plant as a nutrient.

The interaction may, for example, result in the formation of root or stem nodules containing the bacterium, or, by bacterial invasion of lateral roots, in the formation of short, thickened lateral roots (STLRs) which are very similar in appearance to the normal short lateral roots of the plant.

Preferably, the bacterium for use in the method of the invention is oxygen tolerant. By "oxygen tolerant" we include any bacterium which is able to fix atmospheric nitrogen in the presence of at least 0.1% oxygen. Examples of suitably tolerant rhizobia bacteria comprise stem-nodulating rhizobia including Azorhizobium species such as *A caulinodans* (eg. strain ORS571 ATCC deposit No. 43989) and Bradyrhizobium species such as B. ORS310, which normally nodulates stems of *Aeschynomene indicia* (Van Rhiju and Vanderleyden (1995) J. Microbiol Rev. 59: 124-142).

The nitrogenase of *A caulinodans* is tolerant of up to approximately 3% oxygen and the nitrogenase of B. ORS310 is tolerant of up to approximately 0.5% oxygen.

Oxygen tolerant bacteria are preferred because, unlike leguminous plants, non-leguminous plants do not normally contain molecules such as leghaemoglobin which can take up free oxygen to minimize inhibition of nitrogen fixation activity due to inactivation of nitrogenase.

Other bacteria suitable for use in the method of the invention include the Rhizobia which naturally infect legumes (especially temperate legumes) via an infection thread in the root hairs such as *Rhizobium leguminosarum, R. meliloti, R. loti, R. phaseoli, R. japonicum, R. lupini* and *R. trifolii*.

Preferably, Rhizobia used in the methods of the invention are those which disturb the epidermis themselves or which can infect the plant by "crack-

entry", that is, take advantage of a disturbance of the epidermis (for example resulting from physical injury or the emergence of an organ such as a lateral root) and in either case thereby form a nodule. Such Rhizobia include those which naturally form nodules on the roots of *Parasponia* species and those which infect the roots (including lateral roots) or other tropical plants (especially legumes), such as *Aeschynomenes* spp. (for example *A. afraspera*), *Arachis Lypogea, Neptunia oleracea, Stylosanthes* spp. and *Sesbania rostrata*. The Rhizobia also include those which induce nodules on the stems of *Sesbania, Aeschynomenes*, and *Neptunia* species. Rhizobia which infect *Parasponia* and cause nodulation include:

Normal Host	**Examples of strains**
P. rugosa	
P. andersonii	CP283
Cajanus cajan	NGR 70
Centrosema pubescens	
Crotalaria anagyroides	
Flemingia congesta	
Inocarpus fragiferum	
Macroptilium lathyroides	NGR 86, NGR 169
Phaseolus calcaratus	
Stizolobium deeringianum	NGR 179
Stylosanthes gracilis	
Lablab purpuresu	NGR 234
Acacia farnesiana	NGR 71
Leucaena leucocephala	NGR 7, 8, 44, 63, 75, 89, 14/1, 19, 43, 95, 98, 99, 107, 111, 112
Mimosa invisa	} NGR 181, 189, 83, 135
M. pudica	} 114, 190

(from Trinick & Galbraith (1980) New Phytol. 86, 17-26).

The *Parasponia*-infecting *Rhizobium* strains CP283 and 501 are particular examples of effective Rhizobia.

The nod-factor inducing agents useful in the present invention comprise any agent which can induce nod-factors in the bacterium used to inoculate the non-leguminous plant. Preferably, the inducing agents are obtained from plant exudates.

Particularly preferred inducing agents are flavonoids. Dakora, F.D. (1995) Aust. J. Plant Physiol 22, 87-99 provides a review of plant flavonoids.

Flavonoids are naturally occurring plant products which originate from the phenylpropanoid pathway (Table A). Each molecule has a C_{15} skeleton formed from condensation of three malonate units with a phenylalanine-derived C_6-C_3 precursor. Their distribution is widespread in higher plants.

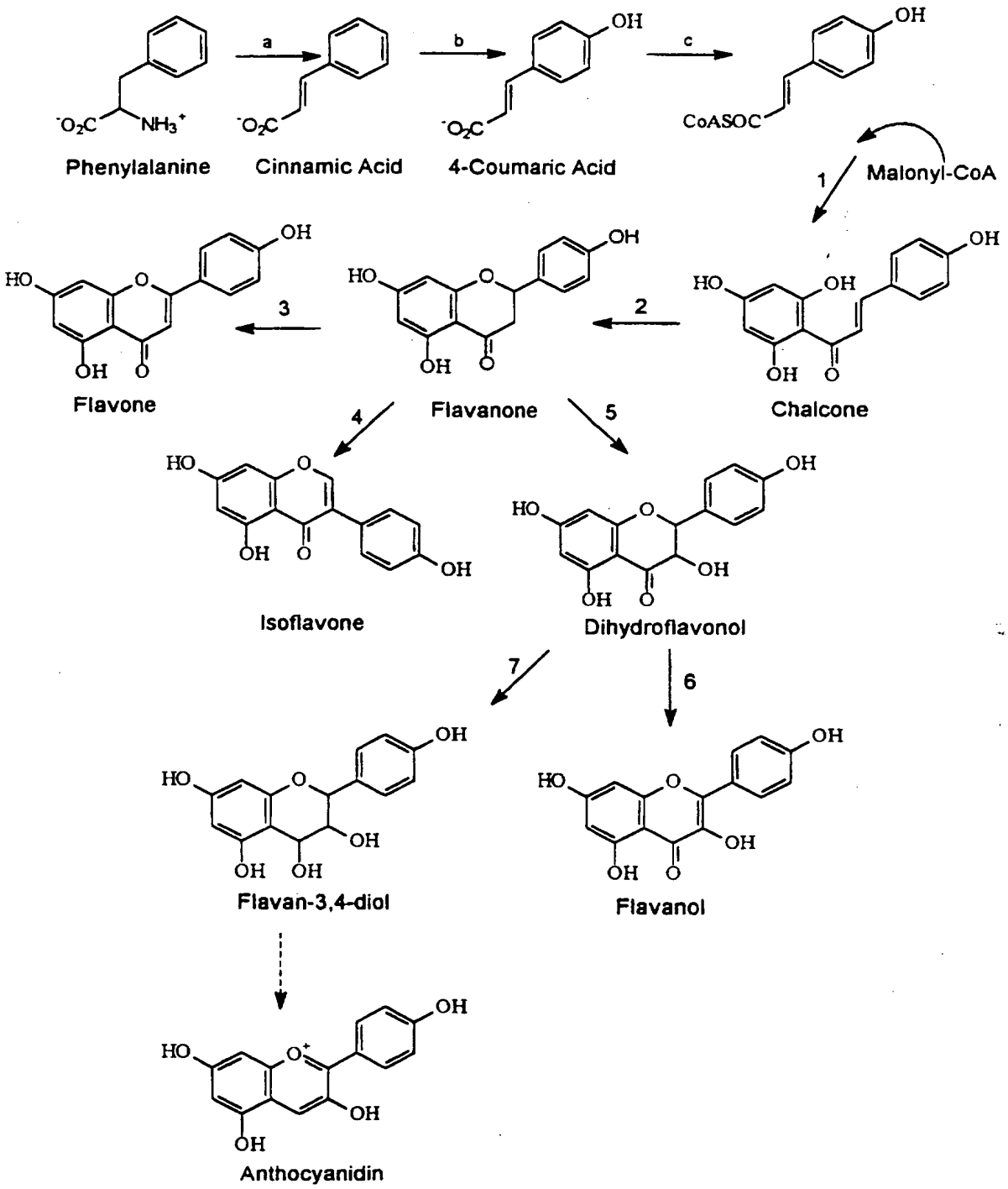

Table A. Proposed pathways of phenylpropanoid metabolism (a-c) and flavonoid biosynthesis (1-7). Enzymes: a, phenylalanine ammonia-lyase (PAL); b, cinnamate 4-hydroxylase: c. 4-coumarate: CoA ligase (4CL): 1, chalcone synthase (CHS); 2, chalcone isomerase (CHI); 3, flavone synthase; 4, 'isoflavone synthase' (possibly two enzymes); 5, flavanone 3-hydroxylate; 6, flavonol synthase; and 7, dihydroflavonol 4-reductase (DFR).

Flavonoid structures, namely flavones, flavanones, isoflavones and chalcones (Table A), induce transcription of nodulation (*nod*) genes in *Rhizobium* cells as the first step towards root nodule formation and symbiotic N_2 fixation. Anthocyanidins such as delphinidin, petunidin and malvidin are also able to transcribe *nod* genes in *Rhizobium leguminosarum* biovar *phaseoli*. The molecules identified are released from seed coats during imbibition and from root exudates of sterile young seedlings. This pool of active molecules is increased in exudate by the release of additional *nod*-gene inducers when rhizobial cells are present on infectible roots.

Table B. Variation in the nodulating signal in different legume-rhizobial symbioses. Harborne (1991): Chapter 11 of "Herbivores: their interactions with secondary plant metabolites" 2 Ed. Vol 1: The Chemical Participants Academic Press. Inc.

Symbiosis	Inducing flavonoids from roots
Rhizobium trifolii on clover	7,4'-dihydroxyflavone 7-methoxy-4'-hydroxyflavone 7,4'-dihydroxy-3'-methoxyflavone
Rhizobium meliloti on alfalfa	5,7,3',4'-tetrahydroxyflavone (luteolin)

Rhizobium leguminosarum on pea	5,7,3',4'-tetrahydroxyflavone (eriodictyol) 5,7,4'-trihydroxyflavone 7-glucoside

Useful flavonoids can be provided as exudate from the seedlings of plants, particularly legumes which normally have nodules on their roots containing the nitrogen fixing bacterium used to inoculate the non-leguminous plant.

Flavonoids can be purified from the exudate of seedlings using conventional methods such as those described by Messens *et al* (1991, Mol. Plant-Microbe Interactions, vol.4, No.3, pp.262-267). They extracted 7,4'-Dihydroxyflavanone (liquiritigenin) as the major nod-factor inducing component of exudate obtained from seedlings of the tropical legume *Sesbania rostrata* which normally includes nitrogen-fixing nodules on its roots and stems containing the bacterium *Azorhizobium caulinodans* strain ORS571. Other flavonoids which were shown by Messens *et al* to imitate the inducing effect of liquiritigenin were 5,7,4'-trihydroxyflavanone (naringenin) and 4,2',4'-trihydroxychalcone (isoliquiritigenin).

Thus, in a preferred embodiment the method of the invention comprises inoculating a non-leguminous plant with Azorhizobium wherein the bacterium is exposed to one or more nod-factor inducing flavonoids selected from naringenin, liquiritigenin and isoliquiritigenin. Preferably, the bacterium is *Azorhizobium caulinodans*.

Non-flavonoid nod-factor inducing agents for use in the invention may include betaines, preferably trigonelline and stachydrine. The latter two betaines were identified by Phillips *et al* (1992) Plant Physiol 99:1526-

1531 as major compounds inducing nod-factor synthesis in seed exudates from the legume Alfalfa.

The inventors have also identified di-iodo 4-hydroxybenzoic acid (Pfaltz Bauer Inc) as a nod-factor inducer in Azorhizobia inoculated wheat seedlings and the related 4-hydroxybenzoic acid as a nod-factor inducer in *Rhizobium* NGR234.

It will be appreciated by a person skilled in the art that the preferred amount of the agent for use in the method of the invention to induce nod-factor production in a particular bacterium and allow it to form a nitrogen-fixing interaction with a particular non-leguminous plant can be determined using routine tests involving nothing more than trial and error.

Preferably, with a sufficient amount of inducing agent the bacterium is exposed to an inducing agent solution having a molarity of from 1×10^{-6} to 5×10^{-4} and especially from 2×10^{-5} to 1×10^{-4}.

Preferably, seedlings of the non-leguminous plants are inoculated with the nitrogen-fixing bacterium in the presence of the nod factor inducing agent shortly after germination. For example, wheat seedlings may be inoculated approximately 3 to 6 days after germination.

Alternatively, the bacterium can be pretreated with the inducing agent prior to inoculation of the plant seedlings.

Preferably, the seeds are sterilised prior to germination and grown under sterile conditions prior to inoculation.

In an alternative preferred embodiment the non-leguminous plant

protoplast is exposed for interaction with the bacterium in the root hairs of the plant without release of the protoplasts from the plant.

Preferably, such exposure is achieved by degrading the plant cell wall at the apices of the root hairs enzymatically.

The bacterium is preferably exposed to the nod-factor inducing agent during or prior to this step.

A suitable experimental protocol for exposing plant protoplasts in accordance with the above preferred embodiment of the invention is described in UK Patent No. GB 2175919B, the disclosure of which is incorporated herein by reference. Particular reference is made to examples 3 and 4 on pages 10 to 12 and example 6 on pages 14 to 16.

It will be appreciated that the procedure described in UK patent GB 2175919B is modified in the present invention in that the bacterium is exposed to a nod-factor inducing agent.

In a further aspect the invention provides a composition comprising a non-leguminous plant seed in combination with a nitrogen-fixing bacterium which is exposed to a nod-factor inducing agent such as a flavonoid.

In the embodiment in which non-leguminous plant seeds are mixed with a plant growth medium such as peat in combination with a bacterial inoculum and a nod-factor inducer, it is preferred that the growth medium is treated with a solution of the inducer. Alternatively, the bacterium can be pre-treated by incubation with the inducer.

Preferably, the composition comprises sterile plant growth medium, such

as peat, mixed with sterilised seeds and the nod-factor inducer together with a bacterial inoculum.

In use, when the plant seed germinates the bacterium invades the emerging roots of the seedlings in the presence of the nod-factor inducing agent to establish a nitrogen-fixing interaction.

Preferably, the composition comprises a non-legume seed or a somatic non-legume plant embryo enclosed within a synthetic coating comprising a growth medium. Such coated seeds are well known, with alginate beads being the most widely studied coating material.

Preferably, the coating comprises a pharmaceutical type capsule as described by Dupuis *et al* (1994) Bio/Technology Vol.12 April pg.385-389 the disclosure of which is incorporated herein by reference. In this example, the capsule body acted as a strong water soluble hull, covered on its inner surface by a watertight film composed of polyvinyl chloride (PVC), polyvinyl acetate (PVA) and bentone as a thickener, to control the nutrient supply and the subsequent development of the somatic embryo. A germination medium and the somatic embryo were then placed in the capsule.

In the present invention a bacterial inoculum is added to the growth medium. The nod-factor inducing agent can also be included in the growth medium, but it is preferably used to induce nod-factor expression in the bacterium before the bacterium is added to the growth medium.

In a further aspect, the invention provides a growth medium for a non-leguminous plant comprising a bacterium capable of fixing nitrogen; wherein the bacterium is exposed to a nod-factor inducing agent. In this

aspect, the medium can be prepared simply by inoculating a known medium with a bacterium which has been pretreated with an inducing agent.

In a further aspect, the invention provides a method of inducing nitrogen fixation in a non-leguminous plant comprising providing a non-leguminous plant or reproduction material of said plant including heterologous genetic material capable of expression to produce a nod-factor inducing agent and inoculating the plant with a nitrogen-fixing bacterium.

Methods have been developed for the production of transgenic wheat, rice, maize and oilseed rape and for most of the other major non-legume crops (S.L. Kothari, *et al.* Transgenic Rice, in Transgenic Plants Vol 2., Edit. Shain-Dow Kung and Ray Wu, p3-20, 1993, Academic Press). The procedures available for the transfer of genes into these major non-legume crops include the use of protoplasts, with direct delivery of the gene by electroporation or chemically induced uptake of the gene followed by regeneration of fertile transgenic plants expressing the introduced foreign gene. Another somewhat similar procedure is to utilise biolistics, the so-called particle gun bombardment method, involving the high velocity microprojectile delivery of the gene(s) into plant cells (without having to remove their cell walls to produce protoplasts) which are capable of regenerating shoots and, ultimately, fertile plants. The bacterium *Agrobacterium tumefaciens* can also be used to deliver genes into cells of the major non-legume crops from which transgenic plants can be regenerated using standard tissue culture procedures. The gene or genes to be inserted for the required flavonoid synthesis and secretion in the root system or target crops will be introduced into the non-legume crops using a plasmid construct containing a suitable promoter, terminator and the gene of interest. The pathway of flavonoid synthesis is well established

in plants (C.J. Beggs, *et al* (1986) Photo control of flavonoid biosynthesis. In "Photomorphogenesis in Plants", Eds. R.E. Kenerick and G.H.M. Kronenberg, pp 467-502 (Martin Nijhoff, Dordrecht) and the failure of any particular non-legume crop to synthesise a specific flavonoid required for activation of interacting nitrogen-fixing bacterium such as rhizobia will be linked to the absence of the required enzyme under the control of a specific gene. This specific "flavonoid synthesis" gene can be produced from legumes by standard molecular procedures involving isolation of messenger RNA and reverse transcription of cDNA followed by introduction into the target non-legume crop plant by delivery into isolated protoplasts, or by incorporating into totipotent cells through biolistics or the use of *Agrobacterium tumefaciens* as the gene vector. The cDNA can be tailored with a suitable promoter (preferably a root-specific promoter) and termination sequence. Putative transgenic non-legume crops would be evaluated at the molecular level for the presence of the "flavonoid synthesis" transgene. Their required flavonoid synthesis capability can be demonstrated using the common acetylene reduction assay for nitrogen fixation (Witty J.F. [1979] Soil. Biol. Biochem. 11: 209-210).

Alternatively, expression of a nod-factor inducing agent by a non-leguminous plant can be provided by blocking a step in a biosynthetic pathway of the plant whereby an intermediate capable of inducing nod-factors accumulates so that the bacterium can be exposed thereto. For example, the conversion of the intermediate naringenin (a nod-factor inducing agent) to Dihydrokaempferol by the enzyme Flavanone-3'-hydroxylate can be blocked by a variety of techniques in which expression of the gene encoding the enzyme is suppressed. Suitable suppression methods are described by Courtney-Gutterson *et al* (1994) Bio/Technology vol.12, pg.268-271 and the disclosure of that document is incorporated herein by reference.

Courtney-Gutterson *et al* (1994) suppressed the expression of the gene for the biosynthetic enzyme chalcone synthase using chimeric anti-sense gene constructs or the introduction of an additional copy of the enzyme-encoding endogenous gene (see Experimental Protocol Section on page 271).

Suitable methods for making transgenic plants capable of expressing flavonoid biosynthetic pathway genes are described in WO90/11682 (DNA Plant Technology Corporation) the disclosure of which is incorporated herein by reference. Also described are methods for suppressing the expression of flavonoid biosynthetic genes.

Methods suitable for introducing exogenous DNA into a plant using Agrobacterium as a vector, whereby the transformed plant is capable of expressing the exogenous DNA, are described in WO92/03041 (Florigene BV) and the disclosure of that document is incorporated herein by reference.

Preferred embodiments of the invention will now be described by way of example with reference to the following figures in which:

Figure 1 shows:

a) Formation of short thickened lateral roots (STLRs; arrowed) on a 21d-old wheat seedling inoculated with *Az. caulinodans* in the presence of 10^{-4}M naringenin.

b) Root of a seedling inoculated with *Az. caulinodans*, without naringenin, which lacks STLRs.

c) Longitudinal section of a STLR from (a) showing crack entry invasion (arrowed), with azorhizobia penetrating between cells at the base of the merging lateral root.

d) The region arrowed in (c) showing azorhizobia between

cells.

e) "Pockets" of azorhizobia, surrounded by fibrillar material, in the intercellular space between root cortical cells.

f) Azorhizobia within a wheat root cortical cell.

AR, azorhizobia; cw, cell wall; cy, cytoplasm.

Figure 2 shows the gene sequence of the chalcone synthase of *Petunia hybrida* (Koes *et al*, 1989, Gene 81, 245-257);

Example 1(a)

Induction of nitrogen fixation in a non-leguminous plant by inoculation with a nitrogen fixing bacterium exposed to a nod-factor inducing agent.

Az caulinodans IRBG314, nod$^-$ V44 Nod A::Tn5 and ORS 571 (ATCC deposit No. 43989) nif$^-$ 57004 (nif04-Tn5) were maintained on YEM medium (Vincent J M. Int. Biological Programme Handbook 15, Oxford. Blackwell Scientific Publications (1970). After 3 d ays (d) of culture in TGYE liquid medium (Ladha J K, *et al*. Appl. & Environ. Microbiol. **55**:454-460 (1989)) the rhizobia were used for inoculation. Seeds of wheat (*Triticum aestivum* var Canon) were surface sterilized [70% v/v ethanol (1 min) followed by 30% v/v Domestos bleach (1h)], washed with sterile water and germinated on water-agar (0.8% w/v; Sigma), 25°C, 16th photoperiod, 250 μmo $s^{-1}m^{-2}$ daylight fluorescent illumination.

After 3d, germinating seedlings were transferred to autoclaved tubes (24 x 200 mm) each containing 25 ml agar-solidified (0.8% w/v; Sigma) N-free Fähraeus medium (Fähraeus G. J. Gen. Microbiol. **16**:374-381 (1957)), one seedling/tube, and grown under sterile conditions. The flavonoid nod-factor inducing agent naringenin (Sigma), from a stock

solution (10 mg ml^{-1} in ethanol), was added to the autoclaved Fähraeus medium, following cooling of the latter to 80°C, to give the required naringenin concentration. *Az. caulinodans* Nod factors P2 fraction (Mergaert P, *et al.* Proc. Natl. Acad. Sci. USA, **90**:1551-1555 (1993) were similarly added from an ethanol stock solution ($1\mu g\ \mu l^{-1}$) to a final concentration of 10^{-6}. After 3 d in tubes, each seedling was inoculated with 0.2ml of the appropriate rhizobial culture ($\sim 10^9$ bacteria ml^{-1}). Nitrogenase activity was determined by the acetylene reduction assay using 10% acetylene with 90% air in tubes closed with subaseals (Witty J F. Soil Biol. Biochem. **11**:209-210 (1979). The number of short thickened lateral roots (STLRs) was determined by direct measurement of the number of lateral roots which were more than 0.5 mm wide at the base and up to 2 mm in length.

Table 1: **Naringenin stimulation of nitrogenase activity and STLR formation in wheat inoculated with *Azorhizobium caulinodans***

Naringenin Molarity	14d		21d		28d	
	Nano moles ethylene/ plant/24h	STLRs	Nano moles ethylene/ plant/24h	STLRs	Nano moles ethylene/ plant/24h	STLRs
0	0	0	0	0	0	0
0*	729.4±561.9	3.3±1.2	N.D	N.D	N.D	N.D
$2x10^{-5}$	143.2±117.8	5.8±2.4	93.9±71.7	4.0±1.4	106	8
$1x10^{-4}$	1802.9±921.6	9.3±4.5	880.2±587.5	14.8±9.5	732.2±371,5	15.6±9.0

N.D. Not Determined ± Standard Deviation

* 10^{-6}M Nod factor (P2 fraction) from Az caulinodans added.

Table 1:

Nitrogenase activity was determined using the acetylene reduction assay at various times post inoculation. Uninoculated seedlings, with and without naringenin, and seedlings inoculated with *Az. caulinodans* nif$^-$ and *Az caulinodans* nod$^-$, with and without naringenin, failed to produce ethylene or STLRs either at 14d, 21d or 28d. Thirty replicates were assayed per treatment. All seedlings inoculated with *Az. caulinodans* in the presence of naringenin showed some positive ethylene production and the formation of STLRs.

Example 1b

Roots were fixed in 2.0% (v/v) glutaraldehyde for 24h at 4°C, followed by 1.0% (w/v) osmium tetroxide (2h, 4°C). Fixatives were prepared in 0.1M sodium phosphate buffer, pH 7.0. Specimens were dehydrated through 10% (v/v) ethanol to absolute ethanol (30 min each) and embedded in LR White medium grade resin (The London Resin Co., Basingstoke, UK) (Davey M R *et al.* J. Exp. Bot. **44**:863-867 (1993). For light microscopy (Fig. 1 c,d) sections were cut to 2 μm on glass knives, collected on glass slides and stained with 0.5% (w/v) toluidine blue in 0.1% (w/v) sodium tetraborate (2 min, 60°C), prior to mounting in styromount (Raymond Lamb, Lond, UK). Ultra-thin sections were collected on Pioloform-coated (Agar Scientific, Stansted, UK) copper grids, stained with lead citrate and examined at 80 kV in a Jeol 100-S transmission electron microscope (Davey M R *et al.* J. Exp. Bot. **44**:863-867 (1993).

Results

A highly stimulatory effect of the flavonoid naringenin on nitrogen

fixation and on the formation of short, thickened lateral roots (STLRs) was observed (Table 1) in wheat inoculated with *Az. caulinodans*, compared with inoculated seedlings without naringenin (compare Fig 1a and b). The finding that a mixture of Nod factors, isolated from *Az caulinodans*, also stimulates nitrogenase activity of inoculated wheat seedlings and STLR formation (Table 1), suggests that the stimulation by naringenin results from the flavonoid inducing nodulation genes for Nod factor synthesis comparable to the situation in the *Azorhizobium-Sesbania* symbiosis. This is further supported by the finding that inoculation with a nod$^-$ strain of *Az. caulinodans*, which fails to produce Nod factors, did not result in nitrogen fixing wheat seedlings, or the formation of STLRs invaded by rhizobia, in seedlings at either 14, 21 or 28 days post inoculation (Table 1). Viewed overall, these results indicate that the formation of lateral roots invaded by crack entry (Fig 1c-f), and the onset of nitrogen fixation in wheat inoculated with azorhizobia, are controlled by the same types of signals that are known to operate during the symbiotic interaction between rhizobia and legumes, allowing symbiotic nitrogen fixation to be established in non-legume crops, such as wheat.

Intercellular infection by azorhizobia in wheat resulting from crack entry (Fig 1c,d) is followed by multiplication of the bacteria forming intercellular infection pockets filled with azorhizobia (Fig 1e). The presence of azorhizobia in wheat cells (Fig 1f) probably results from localised cell wall degradation. In wheat seedlings inoculated with azorhizobia in the presence of naringenin, invaded lateral roots can be readily identified after inoculation by the marked swelling at their bases resulting from this crack invasion (Fig 1c,d). A similar swelling at the base of laterals, resulting in the formation of STLRs, also occurs following inoculation with azorhizobia in the presence of Nod factors from *Az. caulinodans* (Table 1). No STLRs were produced following

inoculation with azorhizobia in the absence of naringenin, or by naringenin without inoculation (Table 1). These observations, coupled with the fact that Nod factors from *Az. caulinodans* are known to induce swellings at the base of lateral roots of the legume *Sesbania rostrata*, suggest that Nod factors, induced in *Az. caulinodans* by naringenin in the above experiment, are controlling the invasion of wheat roots by azorhizobia and the development of nitrogen fixation in the resulting STLRs.

The inventor also observed that naringenin stimulates nitrogen fixation in wheat when other rhizobia, such as *Bradyrhizobium* ORS310 which normally nodulates stems of *Aeschynomene indica* are used for inoculation (data not shown). This suggests that naringenin may be a generally active nod gene inducing signal for various rhizobia interacting with non-legumes, leading to nitrogen fixation.

Nitrogenase activity in *S. rostrata* seedlings inoculated with *Az. caulinodans* under the tube growth conditions mentioned previously, and assayed as in Table 1, was increased from 161 nmoles ethylene/plant/24 h to 689 mnoles ethylene/plant/24 h by 10^{-4}M naringenin. The significant stimulation of nitrogen fixation in inoculated wheat by naringenin (Table 1), coupled with the formation of STLRs invaded by azorhizobia, indicates that the nitrogenase activity is arising from rhizobia within the root system. The fact that wheat seedlings inoculated with the nod$^-$ strain of *Az. caulinodans*, which is capable of nitrogen fixation in the free-living state in the presence of up to 3% oxygen, failed to show nitrogenase activity with and without naringenin, as did the wild-type *Az. caulinodans* used for inoculation without naringenin (Table 1), also indicates that nitrogenase activity is arising from rhizobia within the root system of the wheat seedlings. Moreover, the nitrogen fixation activity of surface azorhizobia would be completely inhibited by the concentration of oxygen

used in the acetylene reduction assay system. The failure of a nif⁻ mutant of *Az. caulinodans* (Table 1) to produce ethylene in the assay for nitrogenase activity demonstrated the absence of azorhizobia stress-induced plant ethylene production.

These results demonstrate that the flavonoid naringenin activates *Az. caulinodans* to produce Nod factors which stimulate the entry of azorhizobia into the wheat root system, inducing the formation of (STLRs). The signalling system operating in wheat interacting with azorhizobia, in the presence of naringenin, may be comparable to that in the legume *Sesbania rostrata* during symbiotic nitrogen fixation with *Az. caulinodans*.

Similar results were obtained with other non-legumes including rice, maize, oil-seed rape and *Arabidopsis thaliana*, which suggests that such naringenin induced stimulation of nitrogen fixation is likely to occur in all non-legumes..

Example 2

Protocol for Induction of Rhizobium Uptake into Non-Legume Root Hairs

Rice seeds (dehusked) are surface sterilised in 30% (v/v) Domestos solution and germinated on nitrogen-free agar at 28°C in the dark. This medium (Fahraeus) has the following composition:- [$CaCl_2$ (0.1 g), $MgSO_4.7H_20$ (0.12 g), KH_2PO_4 (0.1 g), $Na_2HPO_4.2H_2O$ (0.15 g), Fe citrate (0.005 g), traces of: Mn,Cu,Zn,B,Mo] per litre with 0.8% agar, pH 6.5-7.0.

40-48 hour old seedlings are incubated for 5 minutes in isotonic enzyme

mixture of the following composition: [Worthington CEL (1 g), Novozyme 2,3,4 (0.5 g), Pectolyase (0.1 g), mannitol (8.0 g)] per 100 ml, pH 5.6-5.8, then transferred to the *Rhizobium* uptake treatment.

Exponential phase *Rhizobia* are cultivated by centrifugation and resuspension in fresh yeast extract-mannitol solution (0.5 ml) having the composition [K_2HPO_4 (0.5 g), $MgSO_4.7H_20$ (0.2 g), NaC1 (0.1 g), mannitol (10.0 g), yeast extract (Difco)(0.4 g)] per litre, pH 6.8-7.0. Immediately prior to the root immersion this preparation is mixed with 1 ml of polyethylene glycol solution of composition [Polyethylene glycol M.W. 6000 (20.0 g), $CaCl_2.2H_20$ (0.15 g)] per 100 ml [Difco is a Registered TM].

The enzyme-treated roots are immersed in the polyethylene glycol with *Rhizobia* for 5 minutes followed by two washings with mannitol solution [Mannitol (9.0 g) per 100 ml, pH 6.5-7.0].

The seedlings are transferred to nitrogen-free agar in square Petri dishes which can be stacked vertically for optimum seedling growth. All seedlings survive the enzyme treatment although the root growth maybe impaired in some cases. Root samples are removed for light and electron microscopic examination.

Fusion of Protoplasts Extruding from Root Hair Tips or Isolated from Root Hairs with Protoplasts Isolated from other Plant Species

Gene transfer by protoplast fusion is a well established procedure for the transfer of clusters of nuclear or cytoplasmic genes, and the use of protoplasts being released from root hairs now enables the basic strategy of somatic hybridisation to be applied to the intact plant. Such protoplast

fusion using either chemical or electrical procedures (Davey, M.R. and Kumar, A., Int. Rev. Cytol. Suppl. 16, 219-299 (1983)) may be used to transfer nuclear genes (controlling for instance symbiotic associations) or cytoplasmic genes (conveying for instance male sterility) without impairment of the functional integrity of the plant. Exposed plasma membranes readily regenerate a new cell wall and root hairs with exposed plasma membranes, after fusion, may be stabilised in this way. If required, protoplasts can be isolated from root hairs and used for somatic hybridisation by fusion with other isolated protoplasts.

This technique is illustrated in Example 3 below.

Example 3

Protocols for Uptake of Rhizobium into Non-Legume Root Hairs

These procedures are generally applicable to all non-legume crop species in which it is possible to achieve enzymatic degradation of the apices of root hairs. This first procedure involves fusion of protoplasts containing *Rhizobia* (these protoplasts are isolated enzymatically from nodules of the legume) with the exposed plasma membrane of the non-legume root hair whereby the root hair of the non-legume will contain *Rhizobia* in its cytoplasm. The second procedure involves fusion of protoplasts (subprotoplasts) released from the tips of root hairs of enzymatically-treated root hairs of legumes with the exposed plasma membrane of the non-legume root hair; the hybrid root hair on the non-legume then behaves like a legume root hair and interacts with *Rhizobia* in the usual way that legumes do during their normal infection with *Rhizobia*.

Procedure (1)

a) Rice seeds (or seeds of any other non-legume) are surface sterilised in 30% (v/v) Domestos and germinated on nitrogen-free agar (see Example 2) at 28° in the dark, and 2-day-old seedlings are incubated in isotonic enzyme mixture for 5 minutes to expose the plasma membranes at the surface of their root hairs.

b) Protoplasts are isolated from young nodules of the legume using the procedure described by Davey and Cocking, 1973, Nature, 224, 460, which involves incubating the sliced nodule in a cell wall degrading enzyme mixture in a suitable plasmolyticum.

c) Seedlings of the non-legume following the treatment as detailed in (a) are mixed with nodule protoplasts (which contain *Rhizobium*) such that the root hairs are mixed with these nodule protoplasts with a ratio of approximately four nodule protoplasts to every root hair. The root hair system of the non-legume with associated isolated root nodule protoplasts is then incubated in autoclaved 30% w/v polyethylene glycol (PEG) M.W. 6000 containing 0.01 $CaCl_2.2H_20$ and left for 10 minutes at room temperature. The PEG solution is then diluated at 5 minute intervals by the addition of 50% of its volume by nitrogen-free medium (see Example 2, but without agar), and then by the addition of a further 50% of its volume and then by a further 50% of its volume until the PEG has been replaced by this medium. Fusion of the nodule protoplasts with the exposed protoplast of the root hair takes place and will result in the non-legume seedlings possessing root hairs containing *Rhizobia*.

Procedure (2)

a) The procedure is as in (1)(a).

b) Subprotoplasts are isolated from root hairs of the legume by treating root hairs of seedlings with the enzyme mixture under conditions which cause extrusion of the protoplast from the root hairs. The subprotoplasts are collected by flotation.

c) Using PEG as the fusion agent as described in (1)(c) fusion of these root hair subprotoplasts from legumes with the exposed protoplast of the root hair of the non-legume takes place and results in the non-legume seedlings possessing root hairs which resemble physiologically (as far as *Rhizobium* infection is concerned) root hairs of the legume. Such treated seedlings are then incubated with *Rhizobia* of the required legume specificity for infection of the non-legume to take place.

Example 4

Production of compositions of non-legume seeds coated with a growth medium containing a bacterium exposed to a nod-factor inducing agent.

Somatic wheat embryos can be coated with a pharmaceutical type capsule as described by Dupuis *et al* (1994) Bio/Technology, Vol.12 April 385-389.

Pharmaceutical type capsule preparation. The capsules (Elanco Qualicaps®, Lilly, France) consisting of gelatine containing glycerol as a plasticizer, were used without heads. The capsule body was 2cm long

with a 8mm external diameter. It was coated on its inner surface with a layer of an organic solvent solution containing various polymers by pouring this solution inside the capsule and rotating by hand. Before air drying, the excess of mixture was then removed by turning the capsule on end under sterile conditions. The film components used as polymers were PVC (Sigma, France). PVA (Mowilith M70®, Hoechst. Germany) or Poly-DL-Lactid® (Boehringer Ingelheim KG, Germany). Natrosol HHR 250® (Aqualon, France), Tylose® (Hoechst, Germany), Methocel® (SEPPIC) and Bentone SDI® (Eurindis, France) were used as thickeners. After drying, the capsule was filled with 0.5ml of germination medium containing 6g/l of Phytagel® mixed with an inoculum (approx. 0.2ml of a 10^9 ml^{-1} culture medium) of *Az. caulinodans* strain ORS571 which had been pretreated with a 10^{-4} molar solution of the nod-factor inducing agent naringenin.. Finally, a 0.5 to 1.5mm long torpedo shaped embryo was placed on the internal medium without special orientation. As the embryoes converted without arrest into plants, the capsules were placed directly in germination conditions.

Capsule cap. Different methods for closing the capsule can be used. A film cap was obtained by placing in the capsule opening 0.5ml of a liquid polymeric mixture or oil, then subjecting it to air drying (polymers) or lower temperatures (oil). Suitable oils include jojoba oil (Sigma), rape seed oil (Robbe). Where cotton or rockwoll is used as a cap, fibers are then sprayed with silicon before being inserted by hand into the opening.

Example 5

Production of transgenic non-legume capable of producing flavonoids for forming a nitrogen-fixing interaction with a bacterium.

Flavonoid biosynthesis in plants is based on a series of common steps; the key enzyme involved is chalcone synthase which catalyses the synthesis of tetrahydroxy chalcone from coumaryl-CoA and malonyl-CoA. Tetrahydroxy chalcone is readily converted to naringenin by chalcone isomerase of general occurrence in plants. A preferred example of commercially important transgenic non-legume crop is rice, which does not naturally synthesise the *nod* factor inducer naringenin. To make it suitable for use according to the invention, the rice is preferably manipulated genetically to produce naringenin by inserting the gene for chalcone synthase; the apparent inability of rice to produce this flavonoid naturally is probably due to the absence in rice of the chalcone synthase, ChsA gene.

(2) Isolation of chalcone synthase cDNA

A cDNA library was prepared in λgt10 using poly A+RNA isolated from flowers of the petunia (Ausubel *et al.*, 1990 Current Protocols in Molecular Biology. Greene Publishing Associates, New York). Approximately 96,000 plaques of the library were screened for hybridisation to a full length CHS DNA clone of petunia. Hybridisation was carried out under low stringency conditions (2xSSC, 45°C) resulting in the production of high background activity.

Backgrounds were reduced by screening the library with a 33 base pair oligonucleotide (5'-CCTCCAGCAAAGCAACCCTGTTG

GTACATCATG-3' 2xSSC, 55°C). This sequence is located at positions 448-520 in the petunia cDNA CHS clone (Reif *et al.*, 1985 Mol. Gen. Genet. 199: 208-215). EcoRI fragments of positively hybridising lambda clones were subcloned into pBluescriptKS for sequence analysis. Partial sequence analysis of the 5' and 5' ends was performed using primers within the plasmid multiple cloning region. Full sequence analysis was performed by preparing nested deletions (Ausubel *et al.*, 1990).

(3) Insertion of the chalcone synthase gene into *Agrobacterium tumefaciens*

Oligonucleotide mutagenesis was performed to introduce an NcoI site at the start codon and a BamHIsite just after the stop codon for the predicted amino acid sequence so that a perfect fusion could be obtained. The coding sequence was cloned into an expression cassette based on the 35S promoter, [The 35S promoter (from cauliflower mosaic virus) has been fully characterised and is standard in transgenic plant production (Ausubel *et al.*, 1990)] and a 5' untranslated region of a chlorophylla/b binding protein gene of petunia (Cab22L) and the nopaline synthase termination region. This expression construct was introduced into the binary vector pAGS802, which contains a 35S-nptII-ocs construct to confer kanamycin resistance on plant tissue, a lacZα region for ease of cloning and replication origins of ColEI and pVSP (Courtney-Gutterson *et al.*, 1994 Bio/Technology **12**: 268-271). The binary vectors were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation.

(4) Production of transgenic rice expressing and secreting naringenin

This procedure is designed to produce rice plant transgenic for chalcone synthase. No specific root promoter is required since a general expression of the gene in all parts of the plant is desirable to ensure production of naringenin and secretion from the plant (including the roots). A supervirulent strain of *A. tumefaciens* LBA4404 (plG121Hm), containing the chalcone synthase gene; utilising the procedure of Yukoh Hiei *et al.*, 1994 The Plant Journal 6(2): 271-282, was employed. Rice tissues (shoot apices, roots and calli derived from roots) were immersed in the bacterial suspension for several minutes and then transferred without rinsing on to 2N6-AS medium (all tissues except for shoot apices) or N6S3-AS medium (shoot apices), and incubated at 25°C in darkness for 3 days. After the co-cultivation, the materials were rinsed thoroughly with 250 mg l^{-1} cefotaxime in sterile water and placed on 2N6-CH medium. Colonies of cells that had proliferated were plated on a regeneration medium, N6S3-CH, and incubated at 25°C under continuous illumination (about 2000 lux). Regenerated plants (R_0 generation) were eventually transferred to soil in pots and grown to maturity in a greenhouse as described by Yukoh Hiei *et al.*, 1994. The regenerated rice transgenic plants (tested by Southern and Northern analysis) containing and expressing the chalcone synthase gene were screened for secretion of naringenin using the stimulation of nitrogen fixation assay described in Example 1(a).

Details of the gene sequence of the chalcone synthase of *P. hybrida* given in Figure 2 are as follows:

ID PHCHSA standard; DNA; PLN; 4966 BP.
XX
AC X14591;

XX
DT 13-NOV-1991 (Rel. 30; Created)
DT 14-NOV-1991 (Rel. 30, Last updated, Version 8)
XX
DE P.hybrida chsA gene for chalcone synthase
XX
KW chalcone synthase; chsA gene.
XX
OS Petunia hybrida
OC Eukaryota; Plantae; Embryobionta; Magnoliophyta; Magnoliopsida;
OC Asteridae; Solanales; Solanaceae.
XX
RN [1]
RP 1-4966
RA van Tunen A.J.;
RT ;
RL Submitted (07-MAR-1989) to the EMBL/GenBank/DDBJ databases.
RL Van Tunen A.J., Vrije Universiteit, Department of Genetics, de
RL Boelelaan 1087, 1081 HV Amsterdam , The Netherlands.
XX
RN [2]
RP 1109-3973
RX MEDLINE; 90034197.
RA Koes R.E., Spelt C.E., van Den Elzen P.J.M., Mol J.N.M.;
RT "Cloning and molecular characterization of the chalcone synthase
RT multigene family of Petunia hybrida";
RL Gene 81:245-257(1989).
XX
DR EPD; 35055; Ph chalcone synth. A.
DR SWISS-PROT; P08894; CHSA_PETHY.
XX
CC ChsA is the major expressed member of the genefamily in various
CC floral tissues and in seedlings treated with UV light. It is
CC relatively low expressed in tissue culture material
XX
FH Key Location/Qualifiers
FH
FT source 1..4966
FT /organism="Petunia hybrida"
FT /strain="Violet 30"

```
FT                   /tissue_type="leaf"
FT                   /clone_lib="genomic"
FT                   /clone="VIP 17, VIP 71"
FT                   /chromosome="V"
FT   CAAT_signal     1060..1064
FT   TATA_signal     1117..1123
FT   CDS             join(1226..1403,2751..3742)
FT                   /gene="chsA"
FT                   /EC_number="2.3.1.74"
FT                   /product="chalcone synthase"
FT                   /note="pid:g20525"
FT   mRNA            join(1147..1403,2751..one-of(3884^3911))
FT                   /gene="chsA"
FT   exon            1147..1403
FT                   /number=1
FT   intron          1404..2750
FT                   /number=1
FT   exon            2751..one-of(3884^3911)
FT                   /number=2
FT   polyA_signal    3853..3858
FT   polyA_signal    3859..3864
FT   polyA_signal    3882..3888
```

Example 6

Isolation of nod-inducing agents from plants.

Exudate from seedlings or roots of plants such as the legume *Sesbania rostrata* can be subjected to standard purification procedures and the nod-factor inducing components separated and identified to prepare nod-factor inducing agents useful in the method of the invention.

Suitable experimental protocols are described by Messens *et al* (1991) Mol. Plant-Microbe Interactions, Vol.4, No.3, pp.262-267 where standard techniques involving crude exudate fractionation by reversed-phase chromatogrpahy and spectroscopic analysis to identify components

displaying nod-inducing activity in an *Azorhizobium* reporter strain harbouring a nod A::lacZ reporter fusion. The disclosure of Messens *et al* is incorporated herein by reference.

CLAIMS

1. A method of inducing nitrogen fixation in a non-leguminous plant by inoculating the plant with a nitrogen-fixing bacterium, wherein the bacterium is exposed to a nod-factor inducing agent.

2. A method as claimed in Claim 1 wherein the nod-factor inducing agent is provided as an exudate from a plant which normally forms a symbiotic nitrogen-fixing interaction with the bacterium.

3. A method as claimed in Claim 1 or 2 wherein the nod-factor inducing agent can induce nod-factor expression in the bacterium used to inoculate the non-leguminous plant.

4. A method as claimed in Claim 2 or 3 wherein the nod-factor inducing agent is a flavonoid.

5. A method as claimed in Claim 4 wherein the flavonoid is selected from naringenin, liquiritigenin, and isoliquiritigenin.

6. A method as claimed in one of Claims 1 to 5 wherein the bacterium is a species selected from one or more of the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium*.

7. A method as claimed in Claim 6 wherein the bacterium is of the kind which naturally cause nodulation other than solely by infecting root hairs of legumes via an infection thread.

8. A method as claimed in Claim 7 wherein the bacterium is capable of nodulating the stem of a plant.

9. A method as claimed in any one of the preceding claims wherein the bacterium is tolerant of oxygen levels of more than 0.01%.

10. A method as claimed in any one of the preceding claims wherein the bacterium is an *Azorhizobium* species or a *Bradyrhizobium* species.

11. A method as claimed in Claim 10 wherein the bacterium is *Azorhizobium caulinodans*.

12. A method as claimed in any one of Claims 1 to 11 wherein the bacterium is exposed to a solution of the nod-factor inducing agent.

13. A method as claimed in Claim 12 wherein the inducing agent solution has a molarity of from 10^{-6} to 10^{-4}.

14. A method of inducing nitrogen fixation in a non-leguminous plant comprising inoculating the plant with a nitrogen fixing bacterium in the presence of a flavonoid.

15. A method as claimed in Claim 14 wherein the flavonoid is naringenin.

16. A method as claimed in Claim 14 or Claim 15 wherein the bacterium is *Azorhizobium caulinodans*.

17. A method as claimed in any one of Claims 1 to 16 wherein the nod-factor inducing agent is produced by expression of a heterologous genetic material in the plant.

18. Use of a flavonoid in a method comprising inoculating a non-

leguminous plant with a nitrogen-fixing bacterium whereby the bacterium and plant form a nitrogen fixing interaction.

19. Use of a flavonoid as claimed in Claim 18 wherein the flavonoid is naringenin.

20. Use of a flavonoid as claimed in Claim 18 or 19 wherein the bacterium is *Azorhizobium caulinodans*.

21. A non-leguminous plant having a nitrogen-fixing interaction with a bacterium obtainable by a method as claimed in any one of Claims 1 to 17, or a use as claimed in any one of Claims 18 to 20.

22. A nitrogen fixing bacterium which has been made competent to form a nitrogen fixing interaction with a non-leguminous plant, other than a plant of the Parasponia genus.

23. A composition comprising a non-leguminous plant seed mixed with a plant growth medium and an inoculum of a nitrogen-fixing bacterium exposed to an agent capable of inducing nod-factor production in the bacterium.

24. A composition as claimed in Claim 23 wherein the agent is a flavonoid.

25. A plant growth medium for a non-leguminous plant comprising a bacterium capable of fixing nitrogen; wherein the bacterium has previously been exposed to a nod-factor inducing agent.

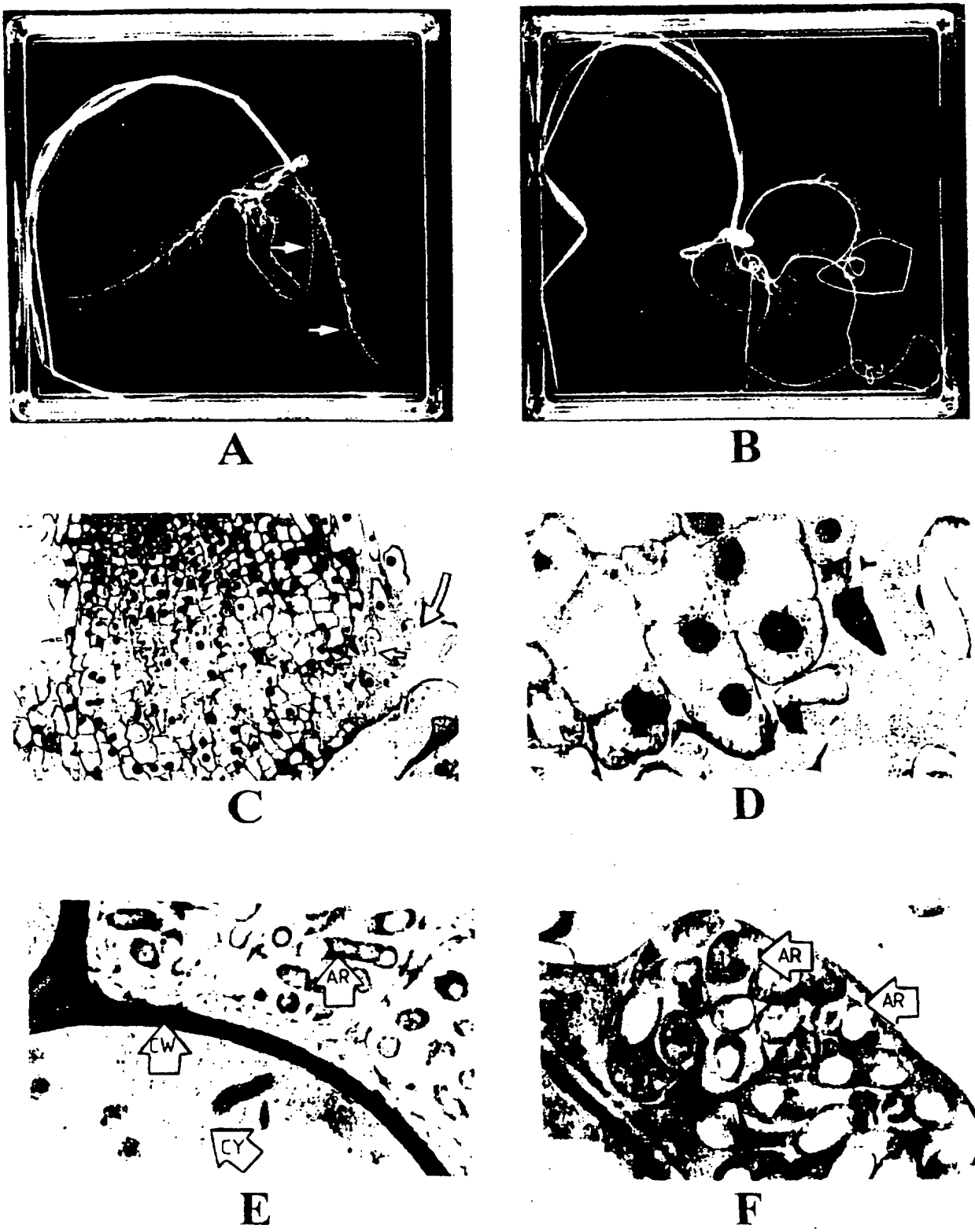

Fig. 1

FIGURE 2

SQ Sequence 4966 BP; 1574 A; 789 C; 853 G; 1746 T; 4 other;

```
aaaaggtgaa gagtttgaaa cgcaaaaaca agcaaaatta acagttttag gctgctttag      60
gtaggaagac acacatttgt ttggaaaata tcttccagaa aatagttctc tcttggcaac     120
tagacatgga aatgaaaatt gagggaaaaa aaaaattaac tgtcttggct aatgatgttt     180
atcgtcgata gtccatatga attagtttct acatcttaga gagtgttgga caataagaca     240
attttgatag aagctaaatt gtagattata ctacatagta tagtcttaat ttctattatg     300
tcaaatcatc aagaagtaaa atactagatg cggaagggta tcgaattcat agcaactggt     360
gtgattcttg aatcagtggt tttcgttttc caaatcttga cgtgagtcgt actcttttt      420
cactgatgat ggatatagta aataaaaccc caatctgatt taggaccata acccttttat     480
agtggaatat aatttatgta gttctaattc agcccatcat taaactagaa ttacatccgg     540
gtatctacac attagcctca tatactttac tttaccagat cactttaatt aggacaacct     600
ttaataataa cagttaacat acattatatt tactttatat atgagcaaat ctatatattt     660
ttacaaattt ttcttctggg atcgaaccaa cacactaatg gcaacttcat aaatatgaca     720
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FIGURE 2 cont

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taactgaaat acttgattta tatatatata tatatatata tatatatata tatatatata   4920
tatatataga ttaatctata tatgatctaa attttttttt tagctc                  4966
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(57) Abstract

The invention relates to a method of inducing nitrogen fixation in non-leguminous plants by inoculating the plant with a nitrogen-fixing bacterium, wherein the bacterium is exposed to a nod-factor inducing agent. The invention also relates to a composition comprising a non-leguminuous plant seed mixed with a plant growth medium and an inoculum of a nitrogen-fixing bacterium exposed to an agent capable of inducing nod-factor production in the bacterium. In another aspect the invention relates to a plant growth medium for a non-leguminous plant comprising a bacterium capable of fixing nitrogen wherein the bacterium has previously been exposed to a nod-factor inducing agent.

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	**Authorized officer** Kania, T

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